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Determining inorganic and organic phosphorus

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Abstract

Phosphorus (P) is a macronutrient for all microalgal species, and the main form of uptake is as orthophosphate (PO_4). In this chapter we present a colorimetric method for determining the PO_4 concentration and dissolved organic phosphorus (DOP) based on total phosphorus (TP) measurements. We also describe a method for determining particulate organic phosphorus (POP) based on the same principles.

Key words: orthophosphate, particulate organic phosphorus, total phosphorus

Running title: Determining phosphorus concentration

1 Introduction

In addition to nitrogen (see separate chapter), phosphorus (P) is the main other nutrient that often limits microalgae growth in natural environments. Algae are in general only able to use orthophosphate (PO_4), but some species are known to be able to use organic forms to some extent [1].

Phosphorus inside the cells can be found, e.g., in the DNA and RNA, and is as such critical for the growth machinery of algal cells. Comparing different algae with different growth characteristics has reviled that the internal ratio of carbon (C): nitrogen (N): phosphorus (P) is lower for species growing rapidly compared with slow-growing species [2], and as such providing more phosphorus than the much used Redfield ratio (106 C :16 N :1 P) might be required to get maximum growth rates.

In this chapter we present a colorimetric method for determining the PO_4 and dissolved organic phosphorus (DOP) based on total phosphorus (TP) measurements [3,4] and a method for determining particulate organic phosphorus (POP) based on the same principles [5]. The determination of TP is based on acid oxidation of organic components with peroxodisulphate in an autoclave, releasing phosphorus as phosphate [3]. POP method involves baking at a high temperature (to decompose organic phosphorus compounds) and hydrolysis of polyphosphate to orthophosphate. Phosphate analysis is based on the antimony-molybdate method. Orthophosphate reacts with molybdate and antimony in an acidic solution to form an antimony-phosphomolybdate complex, which is then reduced by ascorbic acid, forming a blue color. For determining the DOP, there is no direct way as with dissolved organic carbon or nitrogen. Total phosphorus can be used to calculate DOP by subtracting the other P pools (POP could also be removed by first filtering the sample).

2 Materials

Use only analytical grade reagents.

2.1. Phosphate and dissolved organic phosphorus from total phosphorus measurements

1. Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$).
2. Ascorbic acid ($C_6H_8O_6$).
3. Potassium persulphate ($K_2S_2O_8$).
4. Potassium antimony tartrate ($K(SbO)C_4H_4O_6$).
5. Potassium dihydrogen phosphate (KH_2PO_4).
6. Concentrated sulphuric acid (96% H_2SO_4).
7. Concentrated hydrochloric acid (37% HCl).
8. Ultrapure water (e.g., Milli-Q).
9. 50 ml reaction flasks (e.g., Erlenmeyer flasks for manual phosphate analysis).
10. 25-50 ml reaction flasks with screw caps (e.g., Pyrex containers for total phosphorus).
11. 50 ml graduated cylinders.
12. 100 ml volumetric flasks.
13. 100-250 ml storage bottles.
14. Adjustable pipettes and pipette tips.
15. Spatulas.
16. Analytical balance.
17. Refrigerator.
18. Autoclave.
19. Spectrophotometer or automatic analyzer.

20. 1-5 cm quartz glass cuvette (for manual analysis).
21. 0.7 µm glass fiber filters (25 mm Ø) and Swinnex filter holders.
22. 50 ml disposable sterile syringes.
23. Oven (450 ºC).

2.2 Particulate organic phosphorus

1. 0.7 µm glass fiber filters (25 mm Ø).
2. Concentrated sulphuric acid (96% H_2SO_4).
3. Concentrated hydrochloric acid (37% HCl).
4. Sodium sulfate (Na_2SO_4).
5. Magnesium sulfate heptahydrate ($MgSO4 \cdot 7H_2O$).
6. Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$).
7. Ascorbic acid ($C_6H_8O_6$).
8. Potassium antimony tartrate ($K(SbO)C_4H_4O_6$).
9. Potassium dihydrogen phosphate (KH_2PO_4).
10. Ultrapure water (e.g., Milli-Q).
11. 100-1000 ml volumetric flasks.
12. 100-1000 ml storage bottles.
13. 20 glass vials with screw caps (e.g., scintillation vials).
14. Forceps.
15. Spatulas.
16. Adjustable pipette and pipette tips.
17. Oven (450 °C).
18. Vacuum filtration device with a vacuum pump.
19. Analytical balance.

20. Refrigerator.
21. Water bath.
22. Spectrophotometer or automatic analyzer.
24. 2 cm quartz glass cuvette (for manual analyses).

3 Methods

Use acid-washed glassware (soaked for 4 h in 6% HCl and rinsed with deionized water).

3.1. Phosphate

The procedure follows that of Koroleff [4].

1. Prepare 4.5 mol/l sulphuric acid solution. Carefully add 125 ml concentrated acid to 375 ml ultrapure water, cool and make up to 500 ml with ultrapure water. Store in a glass or polyethylene bottle at room temperature.
2. Prepare molybdate solution dissolving 4.75 g ammonium heptamolybdate tetrahydrate in 45 ml ultrapure water, and make up to 50 ml with ultrapure water. Store in a glass or polyethylene bottle at room temperature (usable as long as clear).
3. Prepare tartrate solution by dissolving 1.67 g potassium antimony tartrate in 50 ml ultrapure water. Store in a glass bottle at room temperature (usable as long as clear).
4. Prepare reagent I (acidified ascorbic acid solution) by dissolving 7.0 g ascorbic acid in 65 ml ultrapure water, and then add 35 ml 4.5 mol/l H_2SO_4. Store in an amber glass bottle in a refrigerator (usable as long as colorless).
5. Prepare reagent II (mix reagent) by mixing 22.5 ml molybdate solution to 60 ml 4.5 mol/l H2SO4. Add 2.5 ml tartrate solution and 35 ml ultrapure water. Store in an amber glass bottle at room temperature (stable for months).

6. Prepare phosphate standard 310 µg P/ml by dissolving 0.1361 g potassium dihydrogen phosphate (dried for 2 h at 110°C) in ultrapure water containing 1 ml 4.5 mol/l H_2SO_4, and make up to 100 ml with ultrapure water. Store in a glass bottle in a refrigerator (stable for months).
7. On the day of analysis, prepare suitable working solutions of phosphate (0-310 µg P/l) for calibration by diluting the standard stock solution (see step 6) with ultrapure water (e.g., 0.1 ml to 100 ml for 310 µg P/l solution).
8. Prepare analyzer for analyses according to the operation manual (see Note 1). Dilute the reagents 1:10 for analyzer.
9. Place standards in the autosampler of an analyzer, or measure the standards manually by a spectrophotometer for a calibration curve and linearity test (see Note 2).
10. For manual analyses, measure 35 ml of each calibration standard and 35 ml of ultrapure water for blanks with graduated cylinder to reaction flasks.
11. Add 1 ml of reagent I to standards and the blanks. Mix well by swirling.
12. Add 1 ml reagent II to standards and the blanks. Mix well by swirling and allow reacting for 10 min.
13. Measure the absorbance of standards and blanks in a 5 cm cuvette at 880 nm for a calibration curve (see Note 2).
14. Collect the samples in glass bottles or polyethylene bottles and analyze the samples immediately after collection (within half an hour). The sample can be stored in a glass bottle in a refrigerator for about 2 h.
15. Place samples in the autosampler of an analyzer, or perform manual analyses with a spectrophotometer. Verify proper operation of the instrument with certified reference material, and include ultrapure water blanks in the run (see Note 3).

16. For manual analyses, measure two 35 ml portions of the sample with a graduated cylinder (sample and reference).
17. Add 1 ml of reagent I to both flasks. Mix well by swirling.
18. Add 1 ml reagent II to one flask and 1 ml ultrapure water to the second flask (the reference). Mix well by swirling and allow to react for 10 min.
19. Measure the absorbance of samples in a 5 cm cuvette at 880 nm (if the absorbance is greater than 0.6, dilute the sample or use smaller cuvette) and calculate the phosphate concentration by the calibration curve (see Note 4).

3.2. Dissolved organic phosphorus from total phosphorus measurements

The procedure follows that of Koroleff [4].

1. Prepare 4.5 mol/l sulphuric acid solution (see Sect. 3.1).
2. Prepare oxidizing reagent by diluting 15 ml of 4.5 mol/l sulphuric acid to 100 ml with ultrapure water. Dissolve 5 g potassium peroxodisulphate in the solution. Store in a polyethylene bottle protected from light at room temperature (stable for about one week).
3. Prepare molybdate solution (see Sect. 3.1).
4. Prepare tartrate solution (see Sect. 3.1).
5. Prepare reagent I (ascorbic acid solution) by dissolving 7.0 g ascorbic acid in 100 ml ultrapure water. Store in an amber glass bottle in a refrigerator (stable for at least 1 month, usable as long as almost colorless).
6. Prepare reagent II (mix reagent) (see Sect. 3.1).
7. Prepare phosphate standard 310 µg P/ml (see Sect. 3.1).

8. On the day of analysis, prepare suitable working solutions of phosphate (0-600 µg P/l) for calibration by diluting the standard stock solution (see Sect. 3.1) with ultrapure water (e.g. 0.2 ml in 100 ml for 620 µg P/l solution).
9. Measure 35 ml of each calibration standard and 35 ml of ultrapure water for blanks with graduated cylinder to reaction flasks.
10. Add 2 ml oxidizing reagent solution to the flasks and dissolve by swirling. Close the flasks and place in the autoclave. Autoclave for 30 min and allow to cool to room temperature.
11. Prepare analyzer for analyses according to the operation manual (see Note 1). Dilute the reagents 1:10 for analyzer.
12. Place standards in the autosampler of an analyzer, or measure the standards manually by a spectrophotometer for a calibration curve and linearity test (see Note 2).
13. For manual analyses, add 1 ml reagent I (see step 5) to standards and blanks. Mix well by swirling.
14. Add 1 ml reagent II (see step 6) to standards and blanks. Mix well by swirling and allow to react for 10 min.
15. Measure the absorbance of blanks and standards in a 5 cm cuvette at 880 nm for a calibration curve (see Note 2).
16. Filter the sample with the syringe and syringe filter. Rinse the syringe with the sample before filtering. Prepare filter blank by filtering ultrapure water.
17. Collect the samples in polyethylene bottles and analyze the samples immediately after collection (within 2 h).
18. Measure 35 ml of the sample with a graduated cylinder to reaction flasks.

19. Add 2 ml oxidizing reagent solution to the flasks and dissolve by swirling. Close the flasks and place in the autoclave. Autoclave for 30 min and allow to cool to room temperature.
20. Place samples in the autosampler of an analyzer, or perform manual analyses with a spectrophotometer. Verify proper operation of the instrument with certified reference material, and include ultrapure water blanks in the run (see Note 3).
21. For manual analyses, add 1 ml reagent I (see step 5) to the flasks. Mix well by swirling.
22. Add 1 ml reagent II (see step 6) to the flasks. Mix well by swirling and allow to react for 10 min.
23. Measure the absorbance of samples in a 5 cm cuvette at 880 nm (if the absorbance is >0.6, dilute the sample or use 1 cm cuvette), and calculate the total phosphorus concentration by the calibration curve (see Note 4).
24. The results provide the total phosphorus for the filtered sample. For determining the dissolved organic phosphorus, subtract the PO_4 concentration (see Sect. 3.1).

3.3 Particulate organic phosphorus

The method is a modified version of Solórzano and Sharp [5].

1. Prepare glass fiber filters and glass scintillation vials by acid wash (soak in 6% HCl for 4 h), rinse with ultrapure water, and combust for at 450 °C for 4 h.
2. Prepare sodium sulfate solution (0.17 mol/l) by dissolving 12 g anhydrous Na_2SO_4 in 500 ml ultrapure water. Store in a glass bottle at room temperature (stable for several months).

3. Prepare magnesium sulfate solution (0.17 mol/l) by dissolving 42 g $MgSO4 \cdot 7H_2O$ in 1 l ultrapure water. Store in a glass bottle at room temperature (stable for several months).
4. Place the filter in the filtration device, and apply suction with the vacuum pump.
5. Pipette a known volume of culture through the filter (see Note 5), and for blanks use ultrapure water.
6. Rinse with 4 ml 0.17 mol/l Na_2SO_4.
7. Remove the filter with forceps while the suction is on (see Note 6). Rinse the device with ultrapure water between samples.
8. Place the filter in the glass scintillation vial and dry it (see Note 7). For blanks, place pure filters in scintillation vials.
9. Prepare 4.5 mol/l sulphuric acid solution (see Sect. 3.1).
10. Prepare molybdate solution (see Sect. 3.1).
11. Prepare tartrate solution (see Sect. 3.1).
12. Prepare POP reagent I by dissolving 0.7 g $C_6H_8O_6$ in 100 ml ultrapure water. Store in brown glass bottle in a refrigerator (usable as long as colorless).
13. Prepare POP reagent II (mix reagent; as in Sect. reagents II, except 100 ml 4.5 mol/l H_2SO_4 and no water).
14. Prepare phosphate standard 310 µg P/ml (see Sect. 3.1).
15. Prepare 0.2 mol/l HCl solution. Carefully add 16.6 ml concentrated HCl to 500 ml of ultrapure water, cool, and make up to 1000 ml with ultrapure water. Store in a glass bottle at room temperature (stable for months).
16. Prepare working standards of phosphate (0 - 600 µg P/l) for calibration by diluting the standard stock solution with ultrapure water (e.g. 0.2 ml in 100 ml for 620 µg P/l solution).

17. Pipette 10 ml calibration solution and 10 ml ultrapure water for blanks to scintillation vials, and add 200 µl 0.17 mol/l $MgSO_4$.
18. Standards and blanks are put in warm cabinet at 95 °C until solution has dried completely.
19. Put in oven at 450 °C for 2 h.
20. Add 5 ml 0.2 mol/l HCl and add lids.
21. Place in water bath, 80°C for 30 min, and cool to room temperature.
22. Add 5 ml ultrapure water, and leave for 10 min.
23. Prepare analyzer for analyses according to the operation manual (see Note 1). Dilute the reagents 1:10 for analyzer.
24. Place standards in the autosampler of an analyzer, or measure the standards manually by a spectrophotometer for a calibration curve and linearity test (see Note 2).
25. For manual analyses, add 300 µl POP reagent I and mix by swirling. Add 300 µl POP reagent II. Mix well by swirling and allow to react for 10 min.
26. Measure the absorbance of standards and blanks in a 2 cm cuvette at 880 nm for a calibration curve and linearity test (see Note 2).
27. When measuring samples, start by adding 2 ml 0.017 mol/l $MgSO_4$. Prepare 0.017 mol/l $MgSO_4$ solution by diluting 0.17 mol/l solution with ultrapure water.
28. Put in warm cabinet at 95 °C until solution has dried.
29. Put in oven at 450 °C for 2 h (see Note 8).
30. Add 5 ml 0.2 mol/l HCl and add lids.
31. Place in water bath, 80°C for 30 min, and cool to room temperature.
32. Add 5 ml ultrapure water.

33. Place samples in the autosampler of an analyzer, or perform manual analyses with a spectrophotometer. Verify proper operation of the instrument with certified reference material, and include ultrapure water blanks in the run (see Note 3).
34. For manual analyses, add 300 µl POP reagent I and mix by swirling. Add 300 µl POP reagent II, mix well by swirling, and allow to react for 10 min.
35. Measure absorption of the samples at 880 nm with 2 cm cuvette (if the absorbance is greater than 0.6, dilute the sample), and calculate the particulate organic phosphate concentration by the calibration curve (see Note 4).

4 Notes

1. It is critical to have proper training before running the analyzer. This will depend on the system that you are using, and it is beyond the scope of this chapter to go into the details of the operation of the instrument. We have used Thermo Scientific Aquakem 250 analyzer for phosphate.
2. Calibration curve is prepared by plotting measured absorbance of standards versus standard concentrations (linear regression).
3. Start each sample run with blank(s) and reference material to verify proper operation of the instrument. Analyze samples in sequences of max 20 samples followed by a blank and reference material. Insert also standards in the sequence to to test accuracy and drift during the analytical runs.
4. Determine concentration in the sample by the slope of the calibration curve. Reduce the absorbance of the blank/reference from the absorbance of the sample.
5. There should be a clear color on the filter, but not forming a ‘cake’ of biomass.
6. This is to minimize the water content as much as possible.

7. Make sure to loosely cover the scintillation vials, e.g., with aluminum foil; once the filter is completely dry, close the cap. The dried filter can be stored at room temperature.
8. Any marking will burn off, so they need to be placed in a way that you can keep track of sample numbers; mark again after they have cooled down.

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